

Endothelial cell growth factor is achieved through the application of recombinant DNA technology to prepare cloning vehicles encoding the ECGF protein and procedures are disclosed for recovering ECGF protein essentially free of other proteins of human origin. The product is useful for, among other purposes, diagnostic applications and as potential in the treatment of damaged blood vessels or other endothelial cell-lined structures.

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1 RECOMBINANT HUMAN ENDOTHELIAL CELL GROWTH FACTOR

 This invention relates to recombinant DNA-
directed synthesis of certain proteins. More particularly,
this invention relates to endothelial cell growth
5 factor (ECGF), its recombinant DNA-directed synthesis,
and ECGF's use in the treatment of endothelial cell
damage and/or regeneration.

 Endothelial cell growth factor, referred to
herein as "ECGF", is a mitogen for endothelial cells in
10 vitro. Growth of endothelial cells is a necessary step
during the process of angiogenesis [Maciag, Prog.
Hemostasis-and Thromb., 7:167-182 (1984); Maciag, T.,
Hoover, G.A., and Weinstein, R., J. Biol. Chem., 257:
5333-5336 (1982)]. Bovine ECGF has been isolated by
15 Maciag, et al., [Science 225:932-935 (1984)] using
streptomycin sulfate precipitation, gel exclusion
chromatography, ammonium sulfate precipitation and
heparin-Sepharose affinity chromatography. Bovine ECGF
purified in this manner yields a single-chain polypeptide
20 which possesses an anionic isoelectric point and an
apparent molecular weight of 20,000 [Maciag, supra;
Schreiber, et al., J. Cell Biol., 101:1623-1626 (1985);

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1 and Schreiber, et al., Proc. Natl. Acad. Sci.,
82:6138-6142 (1985)]. More recently, multiple forms of
bovine ECGF have been isolated by Burgess, et al., [J.
5 Biol. Chem. 260:11389-11392 (1985)] by sodium chloride
gradient elution of bovine ECGF from the heparin-Sepharose
column or by reversed-phase high pressure liquid
chromatography (HPLC). The two isolated polypeptides,
designated alpha- and beta-ECGF have apparent molecular
weights of 17,000 and 20,000, respectively. Using this
10 procedure, the bovine ECGF contained in 8,500 ml of bovine
brain extract (6.25×10^7 total units) is concentrated
into a total of 6 ml of alpha-ECGF (3.0×10^6 units) and
3 ml of beta-ECGF (5.2×10^5 units). This is a
9,300-fold purification of alpha-ECGF and 16,300-fold
15 purification of beta-ECGF (Burgess, supra). Recently,
murine monoclonal antibodies against bovine ECGF have been
produced (Maciag, et al., supra) which may be useful in
purifying bovine ECGF in a manner similar to the
monoclonal antibody purification of Factor VIIIC described
20 by Zimmerman and Fulcher in U.S. Patent No. 4,361,509.

In general, recombinant DNA techniques are known. See
Methods In Enzymology. (Academic Press, New York) volumes
65 and 68 (1979); 100 and 101 (1983) and the references
25 cited therein, all of which are incorporated herein by
reference. An extensive technical discussion embodying
most commonly used recombinant DNA methodologies can be
found in Maniatis, et al., Molecular Cloning, Cold Spring
Harbor Laboratory (1982). Genes coding for various
30 polypeptides may be cloned by incorporating a DNA fragment
coding for the polypeptide in a recombinant DNA vehicle,
e.g., bacterial or viral vectors, and transforming a

1 suitable host. This host is typically an Escherichia coli
(E. coli) strain, however, depending upon the desired
product, eukaryotic hosts may be utilized. Clones
5 incorporating the recombinant vectors are isolated and may
be grown and used to produce the desired polypeptide on a
large scale.

Several groups of workers have isolated mixtures
of messenger RNA (mRNA) from eukaryotic cells and employed
a series of enzymatic reactions to synthesize double-
10 stranded DNA copies which are complementary to this mRNA
mixture. In the first reaction, mRNA is transcribed into
a single-stranded complementary DNA (cDNA) by an
RNA-directed DNA poly-merase, also called reverse
transcriptase. Reverse transcriptase synthesizes DNA in
15 the 5'-3' direction, utilizes deoxyribonucleoside
5'-triphosphates as precursors, and requires both a
template and a primer strand, the latter of which must
have a free 3'-hydroxyl terminus. Reverse transcriptase
products, whether partial or complete copies of the mRNA
20 template, often possess short, partially double-stranded
hairpins ("loops") at their 3' termini. In the second
reaction, these "hairpin loops" can be exploited as
primers for DNA polymerases. Preformed DNA is required
both as a template and as a primer in the action of DNA
25 polymerase. The DNA polymerase requires the presence of a
DNA strand having a free 3'-hydroxyl group, to which new
nucleotides are added to extend the chain in the 5'-3'
direction. The products of such sequential reverse
transcriptase and DNA polymerase reactions still possess a
30 loop at one end. The apex of the loop or "fold-point" of
the double-stranded DNA, which has thus been created, is
substantially a single-strand segment. In the third

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1 reaction, this single-strand segment is cleaved with the
single-strand specific nuclease S1 to generate a
"blunt-end" duplex DNA segment. This general method is
applicable to any mRNA mixture, and is described by Buell,
5 et al., J. Biol. Chem., 253:2483 (1978).

The resulting double-stranded cDNA mixture
(ds-cDNA) is inserted into cloning vehicles by any one of
many known techniques, depending at least in part on the
particular vehicle used. Various insertion methods are
10 discussed in considerable detail in Methods In Enzymology,
68:16-18 (1980), and the references cited therein.

Once the DNA segments are inserted, the cloning
vehicle is used to transform a suitable host. These
cloning vehicles usually impart an antibiotic resistance
15 trait on the host. Such hosts are generally prokaryotic
cells. At this point, only a few of the transformed or
transfected hosts contain the desired cDNA. The sum of
all transformed or transfected hosts constitutes a gene
"library". The overall ds-cDNA library created by this
20 method provides a representative sample of the coding
information present in the mRNA mixture used as the
starting material.

If an appropriate oligonucleotide sequence is
available, it can be used to identify clones of interest
25 in the following manner. Individual transformed or
transfected cells are grown as colonies on a nitro-
cellulose filter paper. These colonies are lysed; the DNA
released is bound tightly to the filter paper by heating.
The filter paper is then incubated with a labeled oligo-
30 nucleotide probe which is complementary to the structural
gene of interest. The probe hybridizes with the cDNA for
which it is complementary, and is identified by autoradio-

1 graphy. The corresponding clones are characterized in
order to identify one or a combination of clones which
contain all of the structural information for the desired
protein. The nucleic acid sequence coding for the protein
5 of interest is isolated and reinserted into an expression
vector. The expression vector brings the cloned gene
under the regulatory control of specific prokaryotic or
eukaryotic control elements which allow the efficient
expression (transcription and translation) of the
10 ds-cDNA. Thus, this general technique is only applicable
to those proteins for which at least a portion of their
amino acid or DNA sequence is known for which an oligo-
nucleotide probe is available. See, generally, Maniatis,
et al., supra.

15 More recently, methods have been developed to
identify specific clones by probing bacterial colonies or
phage plaques with antibodies specific for the encoded
protein of interest. This method can only be used with
"expression vector" cloning vehicles since elaboration of
20 the protein product is required. The structural gene is
inserted into the vector adjacent to regulatory gene
sequences that control expression of the protein. The
cells are lysed, either by chemical methods or by a
function supplied by the host cell or vector, and the
25 protein is detected by a specific antibody and a detection
system such as enzyme immunoassay. An example of this is
the lambda gt₁₁ system described by Young and Davis,
Proc. Nat'l. Acad. Sci. USA, 80:1194-1198 (1983) and Young
and Davis, Science, 222:778 (1983).

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1 The present invention has made it possible to
provide readily available, large quantities of ECGF or
ECGF fragments. This has been achieved with oligo-
nucleotides whose design was based upon knowledge of the
5 amino acid sequence of bovine ECGF and which react
specifically with the ECGF cDNA. Production of ECGF is
achieved through the application of recombinant DNA
technology to prepare cloning vehicles encoding the ECGF
protein and procedures for recovering ECGF protein
10 essentially free of other proteins of human origin.

Accordingly, the present invention provides ECGF
or its fragments essentially free of other proteins of
human origin. ECGF is produced by recombinant DNA
techniques in host cells or other self-replicating systems
15 and is provided in essentially pure form. The invention
further provides replicable expression vectors incor-
porating a DNA sequence encoding ECGF and a self-
replicating host cell system transformed or transfected
thereby. The host system is usually of prokaryotic, e.g.,
20 E. coli or B. subtilis, or eukaryotic cells.

The ECGF is produced by a process which comprises
(a) preparing a replicable expression vector capable of
expressing the DNA sequence encoding ECGF in a suitable
host cell system; (b) transforming said host system to
25 obtain a recombinant host system; (c) maintaining said
recombinant host system under conditions permitting
expression of said ECGF-encoding DNA sequence to produce
ECGF protein; and (d) recovering said ECGF protein.
Preferably, the ECGF-encoding replicable expression vector
30 is made by preparing a ds-cDNA preparation representative

- 1 of ECGF mRNA and incorporating the ds-cDNA into replicable
expression vectors. The preferred mode of recovering ECGF
comprises reacting the proteins expressed by the
recombinant host system with a reagent composition
5 comprising at least one binding step specific for ECGF.
ECGF may be used as a therapeutic agent in the treatment
of damaged or in regenerating blood vessels and other
endothelial cell-lined structures.

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Brief Description of the Drawings

Figure 1 illustrates a general procedure for
enzymatic reactions to produce cDNA clones.

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Figure 2 illustrates the production of a library
containing DNA fragments inserted into lambda gt₁₁.

Figure 3 illustrates a partial amino acid
20 sequence of bovine alpha and beta ECGF.

Line a: Amino-terminal amino acid sequence of bovine
alpha ECGF.

Line b: Amino-terminal amino acid sequence of bovine beta
ECGF. The portion in parenthesis corresponds to
25 NH₂-terminal segment for which sequence could
not be determined; amino acid composition is
shown instead. The sequence beginning with
PheAsnLeu... was determined from trypsin-cleaved
bovine beta ECGF.

30 Line c: Amino acid sequence of cyanogen bromide-cleaved
bovine alpha ECGF.

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1 Line d: Amino acid sequence of cyanogen bromide-cleaved
bovine beta ECGF.

Figure 4 illustrates hydrogen-bonded base pairs.

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Figure 5 illustrates the design of an oligonucleotide probe for human Endothelial Cell Growth Factor.

10 Figure 6 illustrates a schematic diagram of human ECGF cDNA clones 1 and 29. The open reading box represents the open reading frame encoding human beta ECGF. The EcoRI sites correspond to synthetic oligonucleotide linkers used in the construction of the
15 cDNA library. The poly (A) tail at the 3' end of clone 1 is shown by A₁₇.

Figure 7 illustrates homology between human ECGF cDNA sequence and oligonucleotide probes.

20 Line a: Bovine trypsin- or cyanogen bromide-cleaved beta ECGF amino acid sequence.

Line b: Unique oligonucleotide probe.

Line c: Human ECGF cDNA sequence (determined from lambda ECGF clones 1 and 29).

25 Line d: Human ECGF amino acid sequence, deduced from cDNA sequence analysis.

Figure 8 illustrates the complete cDNA sequence of human ECGF. The cDNA inserts from ECGF clones 1 and 29
30 were subcloned into M13mpl8 and the ECGF-encoding open reading frame and flanking regions sequenced by the chain termination method. In frame stop codons at the 5' and 3'

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1 ends of the ECGF-encoding open reading frame are indicated
by the underlined sequence and trm, respectively. The
single-letter notation for amino acids is used: A, Ala;
C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K,
5 Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S,
Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

Figure 9 illustrates Northern blot analysis of
ECGF mRNA. RNA was denatured in 2.2 M formaldehyde and
10 50% formamide and fractionated by electrophoresis in a
1.25% agarose gel containing 2.2 M formaldehyde. This was
transferred to GeneScreen Plus (New England Nuclear) by
blotting with 10X SSPE. Blots were hybridized to
32p-labeled nick-translated probes of ECGF clone 1 at
15 65°C for 16 hours in a mixture containing 2X SSPE, 20X
Denhardt's solution, yeast transfer RNA (200 ug/ml), and
0.2% SDS. The membrane was subsequently washed at 65°C,
twice with 2X SSPE and 0.2% SDS, then twice with 0.2X SSPE
and 0.2% SDS, air-dried, and exposed overnight to Kodak
20 XAR film with an intensifying screen. The migration of
28S and 18S RNA is noted.

Lane 1: 10 ug human brain poly(A)-containing RNA.

Lane 2: 10 ug human adult liver poly(A)-containing RNA.

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As used herein, "ECGF" denotes endothelial cell growth factor or its fragments produced by cell or cell-free culture systems, in bioactive forms having the capacity to influence cellular growth, differentiation, and migration in vitro as does ECGF native to the human angiogenic process.

Different alleles of ECGF may exist in nature. These variations may be characterized by differences in the nucleotide sequence of the structural gene coding for proteins of identical biological function. It is possible to produce analogs having single or multiple amino acid substitutions, deletions, additions, or replacements. All such allelic variations, modifications, and analogs resulting in derivatives of ECGF which retain the biologically active properties of native ECGF are included within the scope of this invention.

"Expression vectors" refer to vectors which are capable of transcribing and translating DNA sequences contained therein, where such sequences are linked to other regulatory sequences capable of affecting their expression. These expression vectors must be replicable in the host organisms or systems either as episomes, bacteriophage, or as an integral part of the chromosomal DNA. One form of expression vector which is particularly suitable for use in the invention is the bacteriophage, viruses which normally inhabit and replicate in bacteria. Particularly desirable phage for this purpose are the lambda gt₁₀ and gt₁₁ phage described by Young and Davis, supra. Lambda gt₁₁ is a general recombinant DNA

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1 expression vector capable of producing polypeptides
specified by the inserted DNA.

5 To minimize degradation, upon induction with a
synthetic analogue of lactose (IPTG), foreign proteins or
portions thereof are synthesized fused to the prokaryotic
protein B-galactosidase. The use of host cells defective
in protein degradation pathways may also increase the
lifetime of novel proteins produced from the induced
10 lambda gt₁₁ clones. Proper expression of foreign DNA in
lambda gt₁₁ clones will depend upon the proper orien-
tation and reading frame of the inserted DNA with respect
to the B-galactosidase promoter and translation initiating
codon.

15 Another form of expression vector useful in
recombinant DNA techniques is the plasmid - a circular
unintegrated (extra-chromosomal), double-stranded DNA
loop. Any other form of expression vector which serves an
equivalent function is suitable for use in the process of
this invention.

20 Recombinant vectors and methodology disclosed
herein are suitable for use in host cells covering a wide
range of prokaryotic and eukaryotic organisms. Proka-
ryotic cells are preferred for the cloning of DNA
sequences and in the construction of vectors. For
25 example, E. coli K12 strain HB101 (ATCC No. 33694), is
particularly useful. Of course, other microbial strains
may be used. Vectors containing replication and control
sequences which are derived from species compatible with
the host cell or system are used in connection with these
30 hosts. The vector ordinarily carries an origin of repli-
cation, as well as characteristics capable of providing
phenotypic selection in transformed cells. For example,

1 E. coli can be transformed using the vector pBR322, which
contains genes for ampicillin and tetracycline resistance
[Bolivar, et al., Gene, 2:95 (1977)].

5 These antibiotic resistance genes provide a means
of identifying transformed cells. The expression vector
may also contain control elements which can be used for
the expression of the gene of interest. Common prokaryo-
tic control elements used for expression of foreign DNA
10 sequences in E. coli include the promoters and regulatory
sequences derived from the B-galactosidase and tryptophan
(trp) operons of E. coli, as well as the pR and pL
promoters of bacteriophage lambda. Combinations of these
elements have also been used (e.g., TAC, which is a fusion
of the trp promoter with the lactose operator). Other
15 promoters have also been discovered and utilized, and
details concerning their nucleotide sequences have been
published enabling a skilled worker to combine and exploit
them functionally.

20 In addition to prokaryotes, eukaryotic microbes,
such as yeast cultures, may also be used. Saccharomyces
cerevisiae, or common baker's yeast, is the most commonly
used among eukaryotic microorganisms, although a number of
other strains are commonly available. Yeast promoters
suitable for the expression of foreign DNA sequences in
25 yeast include the promoters for 3-phosphoglycerate kinase
or other glycolytic enzymes. Suitable expression vectors
may contain termination signals which provide for the
polyadenylation and termination of the mRNA transcript of
the cloned gene. Any vector containing a yeast-compatible
30 promoter, origin of replication, and appropriate termi-
nation sequence is suitable for expression of ECGF.

1 Cell lines derived from multicellular organisms
may also be used as hosts. In principle, any such cell
culture is workable, whether from a vertebrate or inver-
tebrate source. However, interest has been greatest in
5 vertebrate cells, and propagation of vertebrate cells in
culture (tissue culture) has become a routine procedure in
recent years. Examples of such useful hosts are the VERO,
HeLa, mouse C127, Chinese hamster ovary (CHO), WI38, BHK,
COS-7, and MDCK cell lines. Expression vectors for such
10 cells ordinarily include an origin of replication, a
promoter located in front of the gene to be expressed, RNA
splice sites (if necessary), and transcriptional termina-
tion sequences.

For use in mammalian cells, the control functions
15 (promoters and enhancers) on the expression vectors are
often provided by viral material. For example, commonly
used promoters are derived from polyoma, Adenovirus 2, and
most frequently, Simian Virus 40 (SV40). Eukaryotic
promoters, such as the promoter of the murine
20 metallothionein gene [Paulakis and Hamer, Proc. Natl.
Acad. Sci. 80:397-401 (1983)], may also be used. Further,
it is also possible, and often desirable, to utilize the
promoter or control sequences which are naturally
associated with the desired gene sequence, provided such
25 control sequences are compatible with the host system. To
increase the rate of transcription, eukaryotic enhancer
sequences can also be added to the construction. These
sequences can be obtained from a variety of animal cells
or oncogenic retroviruses such as the mouse sarcoma virus.

30 An origin of replication may be provided either
by construction of the vector to include an exogenous
origin, such as that provided by SV40 or other viral

1 sources, or may be provided by the host cell chromosomal
replication mechanism. If the vector is integrated into
the host cell chromosome, the latter is often sufficient.

5 Host cells can prepare ECGF which can be of a
variety of chemical compositions. The protein is produced
having methionine as its first amino acid. This methio-
nine is present by virtue of the ATG start codon naturally
existing at the origin of the structural gene or by being
10 engineered before a segment of the structural gene. The
protein may also be intracellularly or extracellularly
cleaved, giving rise to the amino acid which is found
naturally at the amino terminus of the protein. The
protein may be produced together with either its own or a
heterologous signal peptide, the signal polypeptide being
15 specifically cleavable in an intra- or extracellular
environment. Finally, ECGF may be produced by direct
expression in mature form without the necessity of
cleaving away any extraneous polypeptide.

20 Recombinant host cells refer to cells which have
been transformed with vectors constructed using recom-
binant DNA techniques. As defined herein, ECGF is
produced as a consequence of this transformation. ECGF or
its fragments produced by such cells are referred to as
"recombinant ECGF".

25 The procedures below are but some of a wide
variety of well established procedures to produce specific
reagents useful in the process of this invention. The
general procedure for obtaining an mRNA mixture is to
30 obtain a tissue sample or to culture cells producing the
desired protein, and to extract the RNA by a process such

1 as that disclosed by Chirgwin, et al., Biochemistry,
18:5294 (1979). The mRNA is enriched by poly(A)mRNA-
containing material by chromatography on oligo (dT)
cellulose or poly(U) Sepharose, followed by elution of the
5 poly(A) containing mRNA fraction.

The above poly(A) containing mRNA-enriched
fraction is used to synthesize a single-strand
complementary cDNA (ss-cDNA) using reverse transcriptase.
As a consequence of DNA synthesis, a hairpin loop is
10 formed at the 3' end of the DNA which will initiate
second-strand DNA synthesis. Under appropriate
conditions, this hairpin loop is used to effect synthesis
of the ds-cDNA in the presence of DNA polymerase and
deoxyribonucleotide triphosphates.

15 The resultant ds-cDNA is inserted into the
expression vector by any one of many known techniques. In
general, methods can be found in Maniatis, et al., supra,
and Methods In Enzymology, Volumes 65 and 68 (1980); and
100 and 101 (1983). In general, the vector is linearized
20 by at least one restriction endonuclease, which will
produce at least two blunt or cohesive ends. The ds-cDNA
is ligated with or joined into the vector insertion site.

If prokaryotic cells or other cells which contain
substantial cell wall material are employed, the most
25 common method of transformation with the expression vector
is calcium chloride pretreatment as described by Cohen,
R.N., et al., Proc. Nat'l. Acad. Sci. USA, 69:2110
(1972). If cells without cell wall barriers are used as
host cells, transfection is carried out by the calcium
30 phosphate precipitation method described by Graham and Van
der Eb, Virology, 52:456 (1973). Other methods for
introducing DNA into cells such as nuclear injection,

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1 viral infection or protoplast fusion may be successfully
used. The cells are then cultured on selective media, and
proteins for which the expression vector encodes are
produced.

5 Clones containing part or the entire cDNA for
ECGF are identified with specific oligonucleotide probes
deduced from a partial amino acid sequence determination
of ECGF. This method of identification requires that the
non-degenerate oligonucleotide probe be designed such that
10 it specifically hybridizes to ECGF ds-cDNA. Clones con-
taining ECGF cDNA sequences are isolated by radioactively
labeling the oligonucleotide probe with ^{32}P -ATP,
hybridizing the radioactive oligonucleotide probe to the
DNA of individual clones of a cDNA library containing
15 ECGF-cDNA, and detection and isolation of the clones which
hybridize by autoradiography. Such a cloning system is
applicable to the lambda gt₁₁ system described by Young
and Davis, supra.

Clones containing the entire sequence of ECGF are
20 identified using as probe the cDNA insert of the ECGF
recombinants isolated during the initial screening of the
recombinant lambda gt₁₁ cDNA library with ECGF-specific
oligonucleotides. Nucleotide sequencing techniques are
used to determine the sequence of amino acids encoded by
25 the cDNA fragments. This information may be used to
determine the identity of the putative ECGF cDNA clones by
comparison to the known amino acid sequence of the
amino-terminus of bovine ECGF and of a peptide derived by
cyanogen bromide cleavage of ECGF.

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A. Preparation of Total RNA

Total RNA (messenger, ribosomal and transfer) was extracted from fresh two-day old human brain stem essentially as described by Chirgwin, supra, (1979). Cell pellets were homogenized in 5 volumes of a solution containing 4 M guanidine thiocyanate, and 25 mM Antifoam A (Sigma Chemical Co., St. Louis, Mo.). The homogenate was centrifuged at 6,000 rpm in a Sorvall GSA rotor for 15 minutes at 10°C. The supernatant fluid was adjusted to pH 5.0 by addition of acetic acid and the RNA precipitated by 0.75 volumes of ethanol at -20°C for two hours. RNA was collected by centrifugation and dissolved in 7.5 M guanidine hydrochloride containing 2 mM sodium citrate and 5 mM dithiothreitol. Following two additional precipitations using 0.5 volumes of ethanol, the residual guanidine hydrochloride was extracted from the precipitate with absolute ethanol. RNA was dissolved in sterile water, insoluble material removed by centrifugation, and the pellets were re-extracted with water. The RNA was adjusted to 0.2 M potassium acetate and precipitated by addition of 2.5 volumes of ethanol at -20°C overnight.

B. Preparation of Poly(A)-containing RNA

The total RNA precipitate, prepared as described above, was dissolved in 20 mM Hepes buffer (pH 7.2) containing 10 mM EDTA and 1% SDS, heated at 65°C for 10 minutes, then quickly cooled to 25°C. The RNA solution was then diluted with an equal volume of water, and NaCl was added to bring the final concentration to 300 mM NaCl. Samples containing up to 240 A₂₆₀ units of RNA

- 1 were chromatographed on poly(U)-Sephadrose using standard
procedures. Poly(A)-containing RNA was eluted with 70%
formamide containing 1 mM Hepes buffer (pH 7.2), and 2 mM
EDTA. The eluate was adjusted to 0.24 M NaCl and the RNA
5 was precipitated by 2.5 volumes of ethanol at -20°C .

C. Construction of cDNA Clones in Lambda gt_{11}

- The procedure followed for the enzymatic reaction
is shown in Figure 1. The mRNA (20 μg) was copied into
10 ds-cDNA with reverse transcriptase and DNA polymerase I
exactly as described by Buell, et al., supra, and
Wilkensen, et al., J. Biol. Chem., 253:2483 (1978). The
ds-cDNA was desalted on Sephadex G-50 and the void-volume
fractions further purified on an Elutip-D column
15 (Schleicher & Schuell, Keene, NH) following the manu-
facturer's directions. The ds-cDNA was made blunt-ended
by incubation with S1 nuclease [Ricca, et al., J. Biol.
Chem., 256:10362 (1981)]. The reaction mixture consisted
of 0.2 M sodium acetate (pH 4.5), 0.4 M sodium chloride,
20 2.5 mM zinc acetate and 0.1 unit of S1 nuclease per ng of
ds-cDNA, made to a final reaction volume of 100 μl . The
ds-cDNA was incubated at 37°C for one hour, extracted
with phenol:chloroform, and then desalted on a Sephadex
G-50 column as described above.

- 25 The ds-cDNA was then treated with EcoRI methylase
and Klenow fragment of DNA polymerase I using reaction
conditions described in Maniatis, et al., Molecular
Cloning, supra. The cDNA was again desalted on Sephadex
G-50 as described above and then ligated to 0.5 μg of
30 phosphorylated EcoRI linkers using T_4 DNA ligase
(Maniatis, et al., supra). The mixture was cleaved with
EcoRI and fractionated on an 8% acrylamide gel in Tris-

- 1 borate buffer (Maniatis, et al., supra). DNA with a size
greater than 1 kilobase was eluted from the gel and
recovered by binding to an Elutip-D column, eluted with 1
M NaCl and then collected by ethanol precipitation.
- 5 As shown in Figure 2, the DNA fragments were then
inserted into EcoRI cleaved and phosphatase-treated lambda
gt₁₁, using T₄ DNA ligase. A library of 5.7×10^6
phage was produced, of which approximately 65% were
recombinant phage. The library was amplified by producing
10 plate stocks at 42°C on E. coli Y1088 [supE supF metB
trpR hsdR⁻ hsdM⁺ tonA21 strA lacU169 (proC::Tn5)
(pMC9)]. Amplification procedures are described in
Maniatis, et al., supra. Important features of this
strain, described by Young and Davis, supra, include (1)
15 supF (required suppression of the phage amber mutation in
the S gene), (2) hsdR⁻ hsdM⁺ (necessary to prevent
restriction of foreign DNA prior to host modification),
and (3) lacU169 (proC::Tn5), and (4) (pMC9) (a lac
I-bearing pBR322 derivative which represses, in the
20 absence of an inducer, the expression of foreign genes
that may be detrimental to phage and/or cell growth).

D. Identification of Clones Containing ECGF Sequence

- To screen the library for recombinant phage
25 containing ECGF cDNA, 1.5×10^6 phage were plated on a
lawn of E. coli Y1090 [Δ lacU169 proA Δ lon araD139 strA
supF (trpC22::Tn10) (pMC9)] and incubated at 42°C for 6
hours. After the plates were refrigerated overnight, a
nitrocellulose filter was overlaid on the plates. The
30 position of the filter was marked with a needle. The
filter removed after one minute and left to dry at room
temperature. From each plate, a duplicate filter was

- 1 prepared exactly as described, except that the filter was
left in contact with the plate for 5 minutes. All filters
were then prepared for hybridization, as described in
Maniatis, et al., supra. This involved DNA denaturation
5 in 0.5 M NaOH, 1.5 M NaCl, neutralization in 1 M Tris-HCl,
pH 7.5, 1.5 M NaCl, and heating of the filters for 2 hours
at 80°C in vacuo.

To screen the human brain stem cDNA library for
clones containing ECGF inserts, a specific oligonucleotide
10 was designed. This oligonucleotide was based upon a
partial amino acid sequence analysis of the amino terminus
of ECGF. As shown in Figure 3, lines a & b, bovine ECGF
is isolated as two species, designated alpha and beta
ECGF, which differ only in the amino acids found at the
15 respective amino termini. As shown in Figure 3, line b,
beta-ECGF is a slightly larger species than alpha-ECGF.
The exact amino acid sequence at the amino terminus of
beta-ECGF is undetermined, however, a sequence derived
from fast atom bombardment mass spectral analysis and the
20 amino acid composition of the amino terminal tryptic
peptide of bovine beta-ECGF is shown. The amino terminal
blocking group appears to be acetyl. If intact beta-ECGF
is cleaved by trypsin, a second amino amino acid sequence
found in beta but not alpha ACGF starting with
25 PheAsnLeu... is determined. This sequence is also found
at the amino terminus of acidic fibroblast growth factor
[Thomas, K.A: et al., *Proc. Natl. Acad. Sci.*, 82:6409-6413
(1985)]. The amino terminus of alpha-ECGF is AsnTyrLys...
(Figure 3, line a) and is the equivalent of beta-ECGF
30 minus an amino terminal extension. In Figure 3, lines c
and d set forth for comparison the amino acid sequence of
cyanogen bromide-cleaved bovine alpha and beta ECGF,
respectively.

35

1 For oligonucleotide design, the amino acid
sequence IleLeuProAspGlyThrValAspGlyThrLys, corresponding
to alpha-ECGF amino acids 19-29 inclusive, was chosen.
Rather than design a mixture of oligonucleotides covering
5 all of the possible coding sequences (owing to the
degeneracy of the genetic code), a long unique oligo-
nucleotide was designed. Such oligonucleotide probes have
been previously shown to be successful probes in screening
complex cDNA [Jaye, et al., Nucleic Acids Research
10 11:2325-2335, (1983)] and genomic [Gitschier, et al.,
Nature, 312:326-330 (1984)] libraries. Three criteria
were used in designing the ECGF probe: (1) The
dinucleotide CG was avoided. This strategy was based upon
the observed underrepresentation of the CG dinucleotide in
15 eukaryotic DNA [Josse, et al., J. Biol. Chem. 236:864-875,
(1961)]; (2) preferred codon utilization data was used
wherever possible. A recent and comprehensive analysis of
human codon utilization was found in Lathe, J. Mol. Biol.
183:1-12 (1985); and (3) wherever the strategies of CG
20 dinucleotide and preferred codon utilization were
uninformative, unusual base pairing was allowed. This
strategy was based upon the natural occurrence of G:T, I:T,
I:A and I:C base pairs which occur in the interaction
between tRNA anticodons and mRNA codons [Crick, J. Mol.
25 Biol. 19:548-555, (1966)]. A diagram of usual and unusual
base pairs is shown in Figure 4. Use of I (Inosine) in a
hybridization probe was first demonstrated, in a model
experiment, by Ohtsuka, et al., J. Biol. Chem.
260:2605-2608 (1985). The overall strategy and choice
30 made in the design of the oligonucleotide used to screen
the human brain stem cDNA library for ECGF is shown in
Figure 5. In addition, two other oligonucleotides,
designed with the same strategy, were constructed.

- 1 Approximately 30 pmole of the oligonucleotide
shown in Figure 5 were radioactively labeled by incubation
with ^{32}P -gamma-ATP and T4 polynucleotide kinase,
essentially as described by Maniatis, et al., supra.
- 5 Nitrocellulose filters, prepared as described above, were
prehybridized at 42°C in 6X SSPE (1X SSPE = 0.18M NaCl,
0.01M NaH_2PO_4 pH 7.2, 0.001M EDTA), 2X Denhardt's (1X
Denhardt's - 0.02% each Ficoll, polyvinylpyrrolidone,
bovine serum albumin), 5% dextran sulfate, and 100 $\mu\text{g/ml}$
10 denatured salmon sperm DNA. The ^{32}P -labeled oligo-
nucleotide was added following four hours of prehybridi-
zation, and hybridization continued overnight at 42°C .
Unhybridized probe was removed by sequential washing at
 37°C in 2X SSPE, 0.1% SDS.
- 15 From 1.5×10^6 plaques screened, 2 plaques gave
positive autoradiographic signals after overnight
exposure. These clones were purified to homogeneity by
repeated cycles of purification using the above
oligonucleotide as hybridization probe.
- 20 The two clones that were isolated, ECGF clones 1
and 29, were analyzed in further detail. Upon digestion
with EcoRI, clone 1 and 29 revealed cDNA inserts of 2.2
and 0.3 Kb, respectively. Nick translation of cloned cDNA
and its subsequent use as a radiolabeled probe in Southern
25 blot analysis (Maniatis, et al., supra) revealed that
clones 1 and 29 were related and overlapping clones. The
overlapping nature of these two clones is shown in Figure
6.
- 30 Clones 1 and 29 were analyzed in further detail
as follows: An additional two oligonucleotides were
designed, based upon the amino acid sequence of bovine
ECGF. These oligonucleotides were designed based upon the

same considerations as those used in the design of the oligonucleotide used to isolate clones 1 and 29. These oligonucleotides (ECGF oligonucleotides II and III) are shown in Figure 7. These two oligonucleotides as well as oligo(dT)₁₈ were radioactively labeled in a kination reaction as described above and used as hybridization probes in Southern blotting experiments. The results of these experiments showed that the 0.3 Kb cDNA insert of clone 29 hybridized to ECGF oligonucleotides I and II but not to ECGF oligonucleotide III or oligo(dT)₁₈; the 2.2 Kb cDNA insert of clone 1 hybridized to oligonucleotide I, II, III as well as oligo(dT)₁₈. These data and subsequent nucleotide sequence determination of clones 1 and 29 showed that the 3' end of clone 1 ends with a poly(A) tail. Hybridization of clone 1 to ECGF oligonucleotide III, which is based on a cyanogen bromide cleavage product of bovine ECGF, as well as to oligo(dT)₁₈, strongly suggested that this clone contains the rest of the coding sequence for both alpha and beta ECGFs as well as a large (greater than 1 Kb) 3' flanking sequence.

The cDNA inserts from clones 1 and 29 were isolated, subcloned into M13mpl8, and the ECGF-encoding open reading frame and flanking regions sequenced by the chain termination method [Sanger et al., Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977)]. The nucleotide sequence of these clones and the amino acid sequence deduced from the nucleic acid sequence is shown in Figure 8. Examination of the nucleotide sequence reveals an open reading frame of 465 nucleotides encoding human ECGF. The 155 amino acids of human ECGF were found to be flanked by translation stop codons. The NH₂-terminal amino acid of

1 human beta ECGF deduced from the cDNA sequence is
methionine, which most likely serves as the translation
initiation residue. These data, together with the
relatively non-hydrophobic nature of the first 15-20 amino
5 terminal residues, strongly suggest that human beta ECGF
is synthesized without a NH_2 -terminal signal peptide. A
comparison of Figures 3 and 8 shows that the amino
terminal amino acid sequence of trypsin-cleaved bovine
beta ECGF as well as that of bovine alpha ECGF are nearly
10 identical to the amino acid sequence predicted from the
nucleotide sequence of lambda ECGF clones 1 and 29. An
overall homology between the two species of over 95% is
observed.

Northern blot analysis (Maniatis, et al, supra)
15 reveals that ECGF mRNA is a single molecular species which
comigrates with 28S rRNA (Figure 9). Considering the
variation in the estimated size of 28S rRNA, the
approximate size of ECGF mRNA is 4.8 ± 1.4 Kb. All of the
sequence encoding the mature forms of both alpha and beta
20 ECGF is encoded within ECGF clones 1 and 29, which
together encompasses approximately 2.3 Kb. Thus, these
data demonstrate that the region 5' and flanking the
ECGF-encoding sequences, is very large (approximately
 2.5 ± 1.4 Kb).

25 cDNA inserts from clone 1 and clone 29 were
excised by digestion with EcoRI and subcloned in pUC8 at
the EcoRI site. The plasmid formed from clone 1 was
designated pDH15 and the plasmid formed from clone 29 was
designated pDH14. The plasmids were deposited in American
30 Type Culture Collection, 12301 Parklawn Drive, Rockville,
MD 20852. The plasmid from clone 1, pDH15, was designated
ATCC 53336 and the plasmid from clone 29, pDH 14, was
designated ATCC 53335.

1 Thus, this example describes experimental
procedures which provide human endothelial cell growth
factor, essentially free of other proteins of human origin.

5 ECGF has utility in the growth and amplification
of endothelial cells in culture. Currently, ECGF for cell
culture use is extracted from bovine brain by the protocol
of Maciag, et al., [Proc. Natl. Acad. Sci., 76:11,
5674-5678 (1978)]. This crude bovine ECGF is mitogenic
10 for human umbilical vein endothelial cells [Maciag, et
al., J. Biol. Chem 257:5333-5336 (1982)] and endothelial
cells from other species. Utilization of heparin with
ECGF and a fibronectin matrix permits the establishment of
stable endothelial cell clones. The recommended
concentration of this crude bovine ECGF for use as a
15 mitogen in vitro is 150 micrograms per milliliter of
growth medium.

Recombinant DNA-derived human ECGF has utility,
therefore, as an improved substitute for crude bovine ECGF
in the in vitro culturing of human endothelial cells and
20 other mesenchymal cells for research use. The activity of
human ECGF is expected to be the same as or better than
bovine ECGF in the potentiation of endothelial cell growth
due to the high degree of homology in the amino acid
sequences of both proteins. The expected effective dose
25 range for potentiating cell division and growth in vitro
is 5 - 10 ng of purified ECGF per milliliter of culture
medium. Production of the ECGF via recombinant-DNA
technologies as outlined in this patent application and
subsequent purification as described by Burgess, et al.,
30 [J. Biol. Chem. 260:11389-11392 (1985)] will provide large
quantities of a pure product of human origin. (heretofore

1 unavailable in any quantity or purity) with which to
develop models of human homeostatis and angiogenesis.

5 Recombinant DNA-derived human ECGF also has
utility in the potentiation of cell growth on a prosthetic
device, rather than a tissue culture flask or bottle.
This device may or may not be coated with other molecules
which would facilitate the attachment of endothelial cells
to the device. These facilitating molecules may include
10 extracellular matrix proteins (eg. fibronectin, laminin,
or one of the collagens), human serum albumin, heparin or
other glycosaminoclycans or inert organic molecules.
Endothelial cells would be cultured on these surfaces
using effective doses of ECGF in the culture medium,
ultimately covering the device with an endothelial cell
15 monolayer. This device would then provide a
non-thrombogenic surface on the prosthetic device, thus
reducing the risk of potentially life-threatening
thrombogenic events subsequent to implantation of the
prosthetic device.

20 ECGF has utility in diagnostic applications.
Schreiber, et al., [Proc. Natl. Acad. Sci. 82:6138 (1985)]
developed a double antibody immunoassay for bovine ECGF.
In this assay, 96-well polyvinyl chloride plates were
coated with rabbit anti-ECGF and the remaining binding
25 sites subsequently blocked with 10% normal rabbit serum.
Samples of ECGF were then added to the wells and
incubated. After washing, murine monoclonal anti-ECGF was
added. After incubation and several washes, rabbit
anti-mouse IgG coupled with peroxidase was added. The
30 reaction product was quantitated spectrophotometrically
after conversion of O-phenylenediamine in the presence of
hydrogen peroxide. A similarly constructed immunoassay
may be useful for monitoring human ECGF levels in disease

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1 states affecting endothelial cell growth. Purified
recombinant-DNA derived ECGF would be useful as a standard
reagent in quantifying unknown ECGF samples.

5 ECGF also may have potential in the treatment of
damaged or in the regeneration of blood vessels and other
endothelial cell-lined structures.

10 It should be appreciated that the present
invention is not to be construed as being limited by the
illustrative embodiment. It is possible to produce still
other embodiments without departing from the inventive
concepts herein disclosed. Such embodiments are within
the ability of those skilled in the art.

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1 WHAT IS CLAIMED IS:

1. A process for producing human endothelial cell growth factor comprising, providing a replicable expression vector capable of expressing the DNA sequence
5 encoding human endothelial cell growth factor in a suitable host, transforming said host to obtain a recombinant host, and maintaining said recombinant host under conditions permitting expression of said endothelial cell growth factor - encoding cDNA sequence to produce
10 endothelial cell growth factor.
2. The process according to claim 1 including the further step of recovering said endothelial cell growth factor.
- 15 3. The process according to claim 2 wherein said expression vector is a bacteriophage.
4. The process according to claim 3 wherein said
20 bacteriophage is a member of the group consisting of lambda gt₁₀ and lambda gt₁₁.
5. The process according to claim 2 wherein said expression vector is a plasmid.
- 25 6. The process according to claim 5 wherein said plasmid is derived from pBR322.
7. The process according to claim 2 wherein the
30 control function on said expression vector is provided by viral material.

35

- 1 8. The process according to claim 7 wherein said
viral material is a member of the group consisting of
bovine papilloma virus, Epstein Barr virus, adenovirus,
5 Simian virus 40 and baculovirus.
9. Endothelial cell growth factor produced according
to the process of claim 2.
- 10 10. A replicable expression vector capable of
expressing endothelial cell growth factor in a
self-replicating recombinant system.
- 15 11. The self-replicating recombinant system
transformed with the vector of claim 10.
12. The recombinant system according to claim 11
wherein said system is in a cell.
- 20 13. The recombinant system according to claim 11
wherein said system is cell-free.
- 25 14. The recombinant system according to claim 11
obtained by transforming or infecting a member of the
group consisting of an E. coli, B. subtilis, insect, yeast
and vertebrate cell.
15. The recombinant system according to claim 11
obtained by transforming eukaryotes.
- 30 16. The process according to claim 1 wherein
recovering said endothelial cell growth factor comprises
reaction of the proteins expressed by the recombinant host

1

system with a reagent composition comprising at least one binding protein specific for endothelial cell growth factor.

5

17. Human endothelial cell growth factor essentially free of other proteins of human origin.

10

18. The endothelial cell growth factor of claim 17 comprising a polypeptide sequence extending from the NH₂-terminal amino acid of endothelial cell growth factor.

19. A cDNA clone comprising the complete coding sequence for human endothelial cell growth factor.

15

20. A cDNA clone containing untranslated nucleotide sequences located 5' and 3' to the coding sequence of human endothelial cell growth factor as defined by the endothelial cell growth factor mRNA.

20

21. A composition comprising a therapeutically effective amount of human endothelial cell growth factor to promote wound healing in a mixture with an acceptable carrier.

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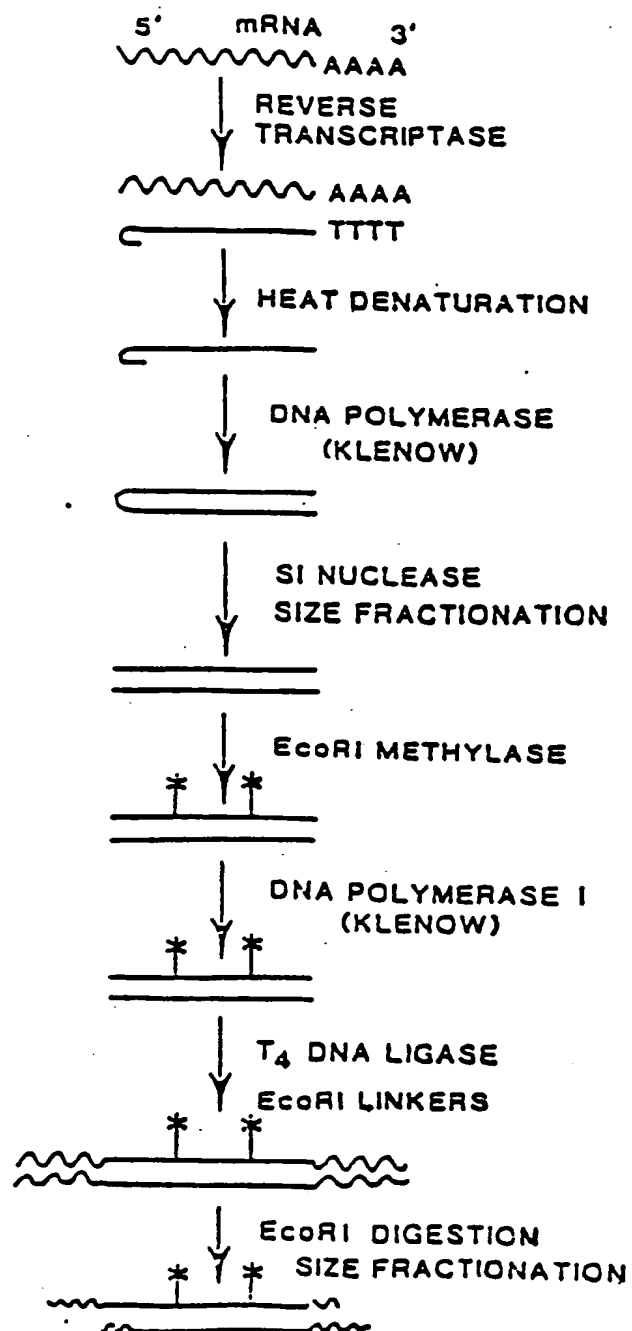


FIG.1

SUBSTITUTE SHEET

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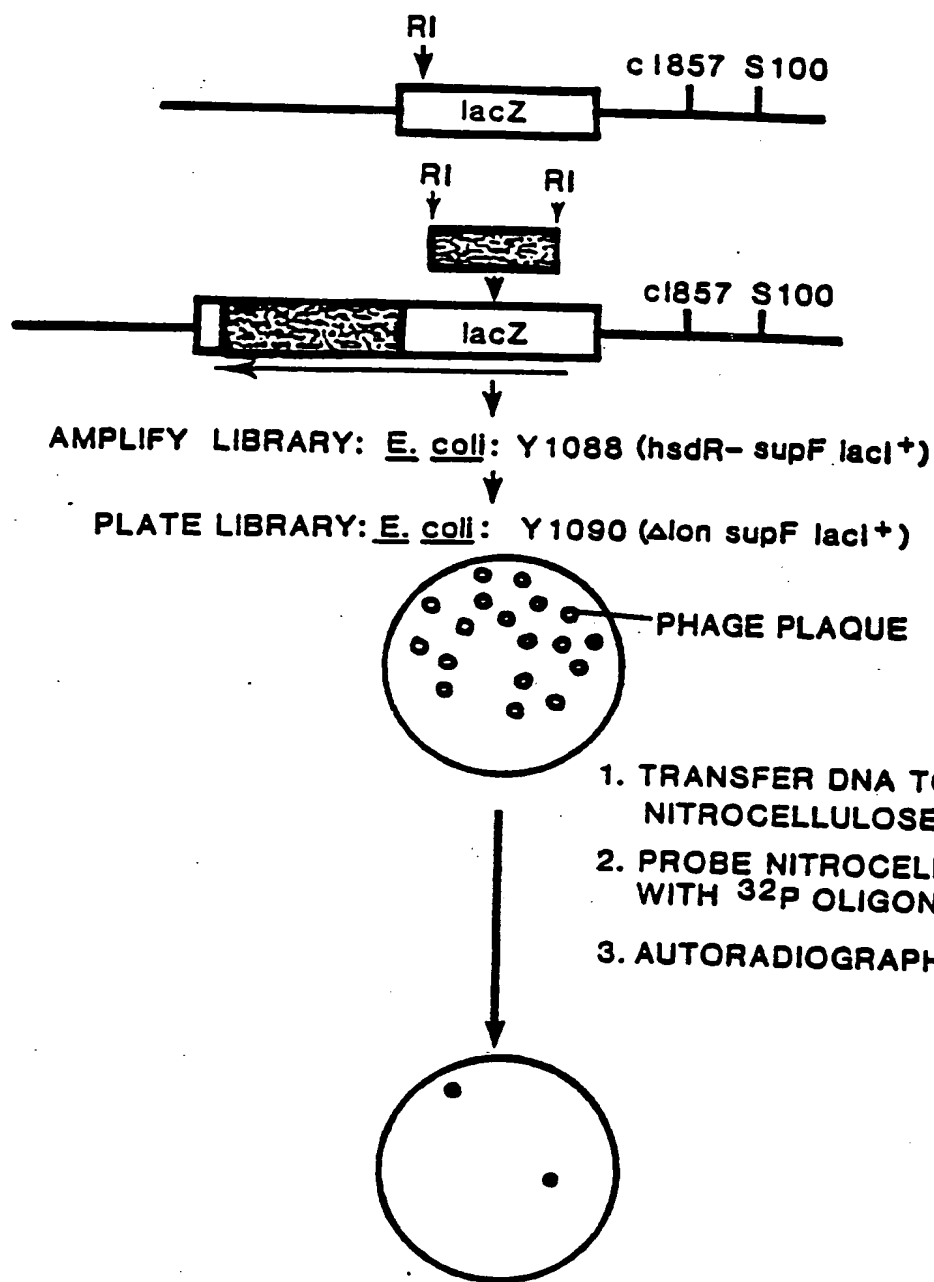


FIG.2

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- 5
(H₂N)-AsnTyrLysLysPro
- a. AcAlaGluGlyGluThrThrThrPheThrAlaLeuThrGluLysPheAsnLeuProLeuGlyAsnTyrLysLysPro
- 10 15 20 25 30
- a. LysLeuLeuTyrCysSerAsnGlyGlyTyrPheLeuArgIleLeuProAspGlyThrValAspGlyThrLysAspAspHis...
- b. LysLeuLeuTyrCysSerAsnGlyGlyTyrPheLeuArgIleLeuProAspGlyThrValAspGlyThrLysAspAspHis...
- c. AspThrAspGluLeuLeuTyrGlySerGlnThrProAsnGluGlu
- d. AspThrAspGluLeuLeuTyrGlySerGlnThrProAsnGluGlu

FIG.3

SUBSTITUTE SHEET

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Hydrogen-Bonded Base Pairs

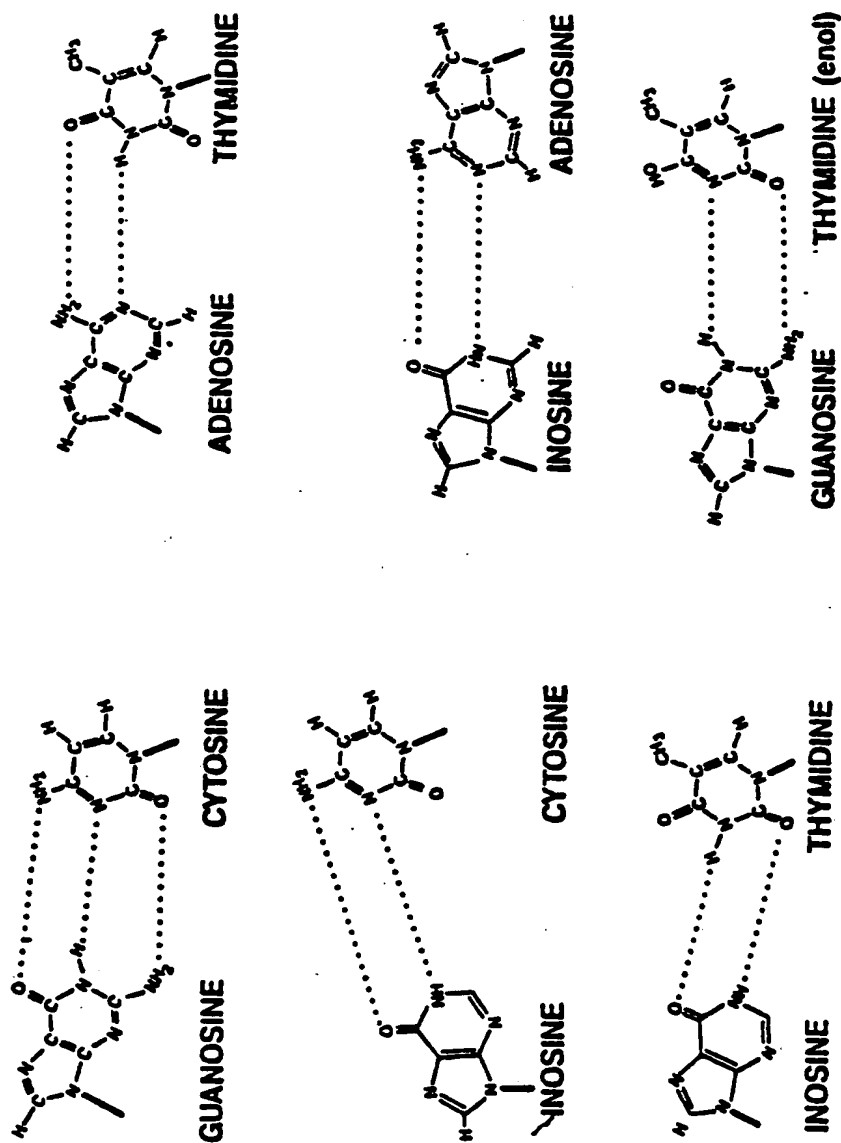


FIG.4

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DESIGN OF AN OLIGONUCLEOTIDE PROBE FOR
HUMAN ENDOTHELIAL CELL GROWTH FACTOR

bovine ECGF

protein sequence:

I L P D G T V D G T K

degenerate coding
sequence:
$$\begin{array}{cccccccccccc} \text{A} & & & & \text{C} & & & & \text{C} & & & & \text{A} \\ \text{AT} & \text{CTX} & \text{CCX} & \text{GA} & \text{GGX} & \text{ACX} & \text{GTX} & \text{GA} & \text{GGX} & \text{ACX} & \text{AA} \\ \text{T} & & & \text{T} & & & & \text{T} & & & \text{G} \\ & \text{C} & \text{TT} & & & & & & & & \end{array}$$

$$3 \times 6 \times 4 \times 2 \times 4 \times 4 \times 4 \times 2 \times 4 \times 2 \times 2 = 2.95 \times 10^5$$

$$\begin{array}{cccccccccccc} \text{A} & & & & \text{C} & & & & \text{C} & & & & \text{A} \\ \text{AT} & \text{CTX} & \text{CCX} & \text{GA} & \text{GGX} & \text{ACX} & \text{GTX} & \text{GA} & \text{GGX} & \text{ACX} & \text{AA} \\ \text{T} & & & \text{T} & & & & \text{T} & & & \text{G} \\ & \text{C} & \text{TT} & & & & & & & & \end{array}$$

CG dinucleotide:

not C or G

not C
or G

not G

not C

not C

$$\begin{array}{cccccccccccc} & & & & \text{C} & & & & & & & & \\ & & & & \text{G} & & & & & & & & \\ \text{A} & & & & \text{A} & & & & \text{C} & & & & \text{A} \\ \text{AT} & \text{CTT} & \text{CCT} & \text{GAT} & \text{GGX} & \text{ACT} & \text{GTX} & \text{GAT} & \text{GGX} & \text{ACT} & \text{AAA} \\ \text{T} & & & & & & & & & & \\ & \text{C} & & & & & & & & & \text{G} \end{array}$$

$$3 \times 8 \times 2 \times 1 \times 4 \times 2 \times 4 \times 2 \times 4 \times 4 \times 2 = 9.84 \times 10^4$$

codon usage:

$$\text{ATT} \begin{array}{c} \text{C} \\ \text{T} \end{array} \text{TT} \begin{array}{c} \text{A} \\ \text{T} \end{array} \text{CCT} \text{GAT} \text{GGX} \begin{array}{c} \text{A} \\ \text{T} \end{array} \text{GTX} \text{GAT} \text{GGX} \begin{array}{c} \text{A} \\ \text{T} \end{array} \text{AAA}$$

$$1 \times 2 \times 2 \times 1 \times 4 \times 2 \times 4 \times 1 \times 4 \times 3 \times 1 = 1.54 \times 10^3$$

allowance for
inosine and

G:T basepairs:

ATT TTT CCI GAT GGI ACI GTI GAT GGI ACI AAA

16	A/T
11	G/C
6	I

33

FIG.5

CHART

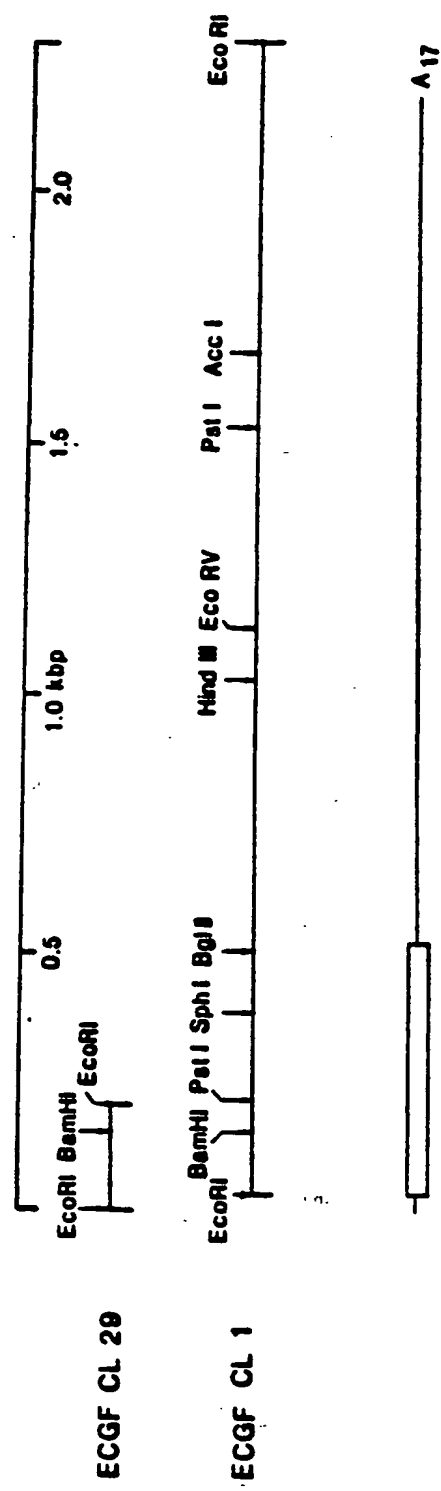


FIG.6

SUBSTITUTE SHEET

a. M D T D G L L Y G S Q T P N E E
b. ATGGACACATGACGGCTCTTACGGITCICAGACTCCIAACGAGGAG
c. : : : : : : : : / : : : : : : : : / : : : : : : : :
d. ATGGACACC6ACGGGCTTTATACGGCTCACAGACACCCAATGAGGA
M D T D G L L Y G S Q T P N E E
ECGF oligonucleotide III*

a. Bovine EC6F protein sequence
b. Oligonucleotide probe
c. Human EC6F cDNA sequence
d. Human EC6F deduced amino acid sequence
: Signifies 6:C or A:T base pairing
/ Signifies unusual base pairing
* Actual oligonucleotide used was the complement of the sequence shown

FIG. 7

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Beta ECGF
 begins here

Acidic FGF
 begins here

Alpha ECGF
 begins here

ECoRI

1
 ATGGCTGAAGGGGAAATCACCACCTTCACAGCCCTGACCGAGAGTTTAAATCTGCTCCAGGGGAATTACAGAAGCCCAAACTCCTCTACTGTAGCAACGGGGGCCACTTCTCTGAGGATC
 H [A] E G E I T T F T A L T E K [F] N L P P G [N] Y K K P K L L Y C S N G G H F L R I

120
 CTTCGGATGGGCACAGTGGATGGGACAGGGGACAGGAGCGACACATTTCAGCTGACGTGCGGAAGCGTGGGGGAGGTGTATATAAGAGTACCGAGACTGGCCAGTACTTG
 L P D G T V D G T R D R S D Q H I Q L Q L S A E S V G E V Y I K S T E T G Q Y L

PstI

360
 GCCATGGACACCGGGCTTTTATACGGCTCACAGACACCAATAGGAATGTTTGTCTCTGGAAAGGCTGGAGGAGAACCATACACACCTATATATCCAAAGAACGATGCAGAGAG
 A M D T D G L L Y G S Q T P N E E C L F L E R L E E N H Y N T Y I S K K H A E K

SphI

480
 AATTGGTTTGTGGCCTCAAGAGAAATGGGAGCTGCAAAACGGGTCCTCGGACTCACTATGGCCAGAAAGCAATCTGTCTCCCTGCGAGTCTCTCTGATTAAAGAGATCTGTTC
 W W F V G L K K N G S C K R G P R T H Y G Q K A I L F L P L P V S S D trm

600
 TGGTGTGACCACCTCCAGAGAGATTTCGAGGGGTCTCACCTGGTTGACCCCAAAAATGTTCCCTTGACCATTTGGCTGCGCTAACCCCCAGCCCCACAGAGGCTGAATTGTGAAGCAACTT

FIG.8

SUBSTITUTE SHEET

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1 2

28S -

18S -

FIG.9

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/00425

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ³ According to International Patent Classification (IPC) or to both National Classification and IPC IPC(4): C12P 21/00, 21/04; C12N 15/00, 1/00, 5/00, 1/20, 1/16 C07K 7/64, 15/00; A61K 37/00						
II. FIELDS SEARCHED <div style="text-align: center; margin-top: 10px;">Minimum Documentation Searched ⁴</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%; text-align: left; border-bottom: 1px solid black;">Classification System</th> <th style="text-align: left; border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="padding: 5px;">U.S.</td> <td style="padding: 5px;">435, 68, 71, 172.3, 240, 253, 255, 317; 935/13; 530/399; 424/177; 514/8, 25</td> </tr> </table> <div style="text-align: center; margin-top: 10px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵</div>			Classification System	Classification Symbols	U.S.	435, 68, 71, 172.3, 240, 253, 255, 317; 935/13; 530/399; 424/177; 514/8, 25
Classification System	Classification Symbols					
U.S.	435, 68, 71, 172.3, 240, 253, 255, 317; 935/13; 530/399; 424/177; 514/8, 25					
COMPUTER SEARCH, CAS, BIOSIS: ENDOTHELIAL CELL GROWTH FACTOR						
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴						
Category ⁶	Citation of Document, ¹⁴ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸				
Y X	<u>The Journal of Biological Chemistry Vol. 257, issued May 25, 1982 (Baltimore Maryland, USA), (MACIAG ET AL), "High and Low Molecular Weight Forms of Endothelial Cell Growth Factor," pages 5333-5336.</u>	1-21 9,17,18				
Y X	<u>The Journal of Biological Chemistry Vol. 260, issued September 25, 1985 (Baltimore Maryland, USA), (BURGESS ET AL), "Multiple Forms of Endothelial Cell Growth Factor," pages 11389-11392.</u>	1-21 9,17,18				
Y X	<u>Biochemistry and Biophysical Research Communications Vol. 124, issued October 15, 1984, (New York, USA), (CONN ET AL), "The Isolation and Purification of Two Anionic Endothelial Cell Growth Factors From Human Brain," pages 262-268.</u>	1-21 9,17,18				
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁵ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Δ" document member of the same patent family</p> </div> </div>						
IV. CERTIFICATION						
Date of the Actual Completion of the International Search ⁹ 15 April 1987	Date of Mailing of this International Search Report ² 30 APR 1987					
International Searching Authority ¹ ISA/US	Signature of Authorized Officer ¹⁰ Alvin E. Tanenholtz					

- | | | |
|---|--|------|
| Y | The Journal of Biological Chemistry Vol. 256 issued August 10, 1981 (Baltimore Maryland, USA), (HEWICK ET AL), "A Gas-Liquid Solid Phase Peptide and Protein Sequenator," pages 7990-7997. | 1-21 |
| Y | J. Mol. Biol. Vol. 183 issued 1985, (London England), (LATHE), "Synthetic Oligonucleotide Probes Deduced from Amino Acid Sequence Data, Theoretical and Practical Considerations," pages 1-12. | 1-21 |

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ^{1*}

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter ^{1,2} not required to be searched by this Authority, namely:

2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ^{1,2}, specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹¹

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ^{1*} with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
Y	<u>Proc. Natl. Acad. Sci. USA</u> Vol. 80 issued March 1983 (Washington, D.C.), (YOUNG ET AL), "Efficient isolation of genes using antibody probes," pages 1194-1198.	1-21
Y	<u>Nucleic Acids Research</u> Vol. 11 issued 1983, (Oxford England), (JAYE ET AL), "Isolation of a human anti-haemophilic factor IX cDNA clone using a unique 52-base synthetic oligonucleotide probe deduced from the amino acid sequence of bovine factor IX, pages 2325-2335.	1-21